Cell, Vol. 94, 229–238, July 24, 1998, Copyright ©1998 by Cell Press

Impaired Viability and Profound Block in Thymocyte Development in Mice Lacking the Adaptor Protein SLP-76

Vadim Pivniouk,* Erdyni Tsitsikov,* Paul Swinton,* Gary Rathbun,† Frederick W. Alt,† and Raif S. Geha*‡

*Children's Hospital and Department of Pediatrics
Harvard Medical School
Boston, Massachusetts 02115
†Center for Blood Research
Howard Hughes Medical Institute
Harvard Medical School
Boston, Massachusetts 02115

Summary

The adaptor protein SLP-76 is expressed in T lymphocytes and myeloid cells and is a substrate for ZAP-70 and Syk. We generated a SLP-76 null mutation in mice by homologous recombination in embryonic stem cells to evaluate the role of SLP-76 in T cell development and activation. SLP-76-deficient mice exhibited subcutaneous and intraperitoneal hemorrhaging and impaired viability. Analysis of lymphoid cells revealed a profound block in thymic development with absence of double-positive $CD4^+8^+$ thymocytes and of peripheral T cells. This block could not be overcome by in vivo treatment with anti-CD3. V-D-J rearrangement of the TCRβ locus was not obviously affected. B cell development was normal. These results indicate that SLP-76 collects all pre-TCR signals that drive the development and expansion of double-positive thymocytes.

Introduction

The T-cell antigen receptor (TCR)/CD3 complex plays a central role in T cell development and activation. T cells can be divided into two subsets based on the structure of their TCR. In the adult, most T cells express a TCR heterodimer consisting of α and β chains, whereas a minor population expresses an alternative TCR consisting of γ and δ chains. Each of these four TCR chains includes a clonally variable (V) region. During intrathymic development, TCR V regions are assembled via a series of site-specific DNA recombinations. For instance, β chain V genes are formed through random recombination of single elements from among three discrete libraries of gene segments, denoted variable (Vβ), diversity (Dβ), and joining (Jβ). Similarly, α chain V genes are assembled randomly from Vα and Jα libraries of gene segments.

Most αβ T lymphocytes undergo differentiation and maturation within the thymus, where the stages of thymocyte development correlate well with the expression pattern of CD4 and CD8 coreceptor molecules. The most immature thymocytes reside in the $CD4^-8^-$ double-negative (DN) population, which makes up only 2%–4% of all thymocytes. DN thymocytes can be further subdivided into four discrete stages defined by the differential expression of the interleukin-2 receptor α chain (CD25) and CD44 (Pgp-1). The sequence of development of DN thymocytes is as follows: $CD25^-CD44^+ \rightarrow CD25^+CD44^+ \rightarrow CD25^+CD44^- \rightarrow CD25^-CD44^-$ cells (Godfrey et al., 1993). At the $CD25^+CD44^-$ stage, DN thymocytes rearrange their TCRβ locus and become subject to a selection event that allows only those cells that successfully rearrange a functional TCRβ chain to arrest further rearrangement at the β locus (β allelic exclusion), expand, and differentiate into double-positive (DP) thymocytes, which account for ~80% of thymic cells (von Boehmer and Fehling, 1997). Cells that reach the DP stage rearrange their TCRα loci, express an αβTCR on their surface, and undergo negative selection. DP thymocytes that survive this process shut off either CD4 or CD8 expression, depending on the specificity of their TCR, and give rise to single-positive (SP) T lymphocytes, which then leave the thymus and populate the peripheral lymphoid organs (Shortman and Wu, 1996).

The selective expansion of $CD25^+CD44^-$ DN cells with productive TCRβ rearrangements is mediated by signals delivered through the pre-TCR, which consists of a conventional TCRβ chain, a monomorphic pre-TCRα (pTα) chain, and components of the CD3 complex (von Boehmer and Fehling, 1997). Mice with targeted disruption of genes encoding components of the pre-TCR exhibit severe defects in T cell development. For instance, mice deficient in the recombination activation genes *RAG-1* or *RAG-2* (Mombaerts et al., 1992b; Shinkai et al., 1992b), which cannot rearrange their TCR loci, or mice deficient in CD3ϵ (Malissen et al., 1995), have arrested T cell development at the DN stage and lack DP thymocytes. Similar, although less severe, defects are found in pTα-, CD3ζ-, and TCRβ-deficient mice (Mombaerts et al., 1992a; Malissen et al., 1993; Fehling et al., 1995).

The important role of the TCRβ chain in thymic development is evidenced by the observation that introduction of a productive TCRβ transgene in RAG-deficient mice restores T cell maturation up to the DP stage (Shinkai et al., 1992a; Mombaerts et al., 1994). The requirement for TCRβ chain expression in the progression to the DP stage can be overcome in RAG-deficient mice by injection of anti-CD3ϵ monoclonal antibodies (MAbs) (Shinkai and Alt, 1994). Therefore, under physiological conditions, the pre-TCR complex is likely to signal via its CD3 subunit. Activation of the protein tyrosine kinase Lck is implicated in signaling through the pre-TCR, since mice lacking Lck (Molina et al., 1992) or expressing a kinase-defective *lck* transgene (Levin et al., 1993) exhibit a defect in early T cell development similar to that seen in TCRβ- and pTα-deficient mice. Lck phosphorylates tyrosine residues within the immune tyrosine activation motifs (ITAMs) of CD3 chains (Cantrell, 1996). Once phosphorylated, ITAMs recruit ZAP-70 to the activated receptor through a high-affinity interaction that involves both src homology 2 (SH2) domains of ZAP-70 (da Silva et al., 1997b). This facilitates subsequent phosphorylation and activation of ZAP-70 by Lck or Fyn. One of the

‡To whom correspondence should be addressed.

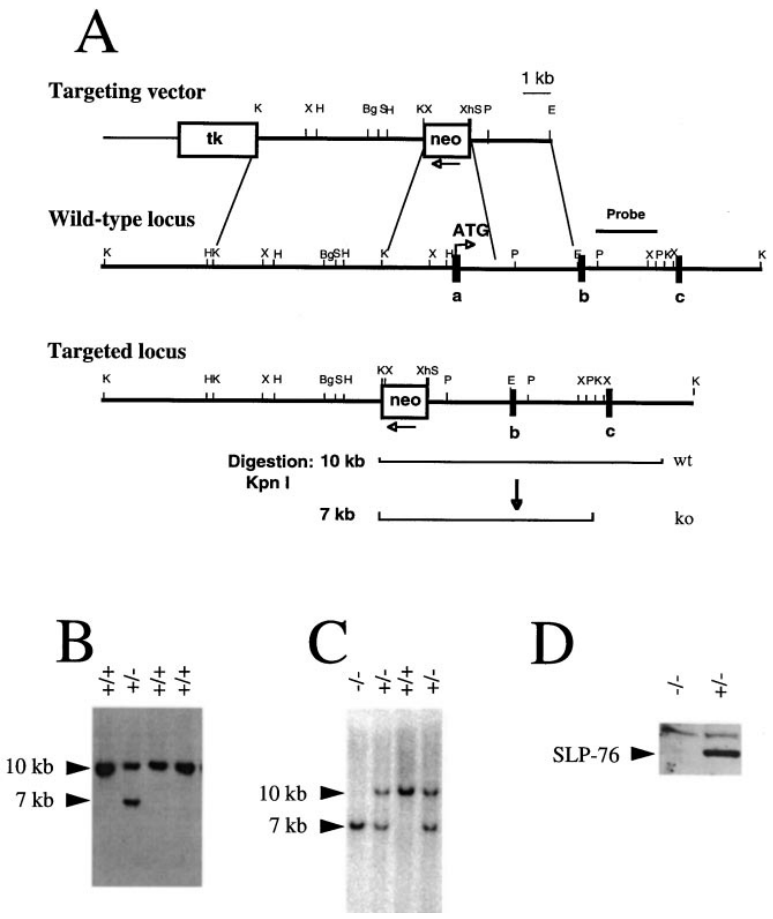

Figure 1. Generation of SLP-76-Deficient Mice

(A) The pSLP-NT targeting construct, the genomic structure of the 5′ end of the *SLP-76* gene, and the predicted structure of the targeted allele after homologous recombination. Exons are represented by black boxes. The precise exon–intron structure has not been determined. neo, neomycin resistance gene; tk, thymidine kinase gene. The 2 kb PstI/PstI hybridization probe is shown. Bg, BglII*; H, HindIII*; E, EcoRI*; K, KpnI; P, PstI*; S, SalI; X, XbaI; and Xh, XhoI (* indicates not all restriction sites are shown). (B) Southern blot analysis of DNA from ES cell clones. Genomic DNA was digested by KpnI and probed with the 2 kb PstI fragment immediately 3′ to the targeted locus, shown in (A). The WT allele is represented by the 10 kb band. The knockout allele is represented by the 7 kb band.

(C) Southern analysis of tail DNA from 3-week-old littermates from brother–sister mating of heterozygous *SLP-76*$^{+/-}$ mice.

(D) Western blot analysis of SLP-76 protein from thymocytes of *SLP-76*$^{-/-}$ and CD25^{+} thymocytes of *SLP-76*$^{+/-}$ littermates. *SLP-76*$^{+/-}$ CD25^{+} thymocytes were used as a control because more than 90% of *SLP-76*$^{-/-}$ thymocytes are CD25^{+}.

substrates of activated ZAP-70 is the adaptor protein SLP-76 (SH2 domain containing leukocyte protein with relative molecular mass of 76 kDa) (Wardenburg et al., 1996; da Silva et al., 1997a; Raab et al., 1997). SLP-76 was originally identified as a molecule that undergoes rapid tyrosine phosphorylation following ligation of the TCR and associates with Grb2 (Jackman et al., 1995). Sequence analysis of SLP-76 reveals three domains: (1) an N-terminal acidic region with several tyrosine residues within consensus motifs for SH2 binding, which in its phosphorylated state binds Vav (Tuosto et al., 1996); (2) a central proline-rich region that binds SH3 domain of Grb2; and (3) a C-terminal SH2 domain that interacts with the Fyn-binding protein also known as a SLP-76-associated phosphoprotein of 130 kDa (FYB or SLAP-130) (da Silva et al., 1997a; Musci et al., 1997) and other proteins, yet to be identified, that include a Ser/Thr kinase and a 62 kDa protein (Koretzky, 1997). SLP-76 is expressed in T cells and in myeloid cells (Jackman et al., 1995; Robinson et al., 1996). Overexpression of SLP-76 results in marked augmentation of TCR-stimulated interleukin-2 (IL-2) and NFAT (nuclear factor of activated T cells) promoter activity and in increased TCR-stimulated IL-2 production (Motto et al., 1996), suggesting that SLP-76 plays an important role in linking proximal TCR signaling to distal events in T cell activation.

By virtue of its interaction with Vav and Grb2, SLP-76 may be coupled to important pathways in T cell activation. Vav was shown to act in a phosphotyrosine-dependent manner as a guanine nucleotide exchange factor (GEF) for the Rac-1 GTPase that activates the JNK (Jun amino-terminal kinase) pathway (Crespo et al., 1997). Vav plays a critical role in T cell development and activation. Vav-deficient mice have impaired T cell development with defective TCR signaling (Tarakhovsky et al., 1995; Zhang et al., 1995; Turner et al., 1997), and overexpression of Vav in T cells leads to basal activation of NFAT and IL-2 transcriptional activity (Wu et al., 1996). SLP-76 may be coupled through Grb2 and Sos to the Ras/mitogen-activated kinase (MAPK)/extracellular signal–regulated kinase (ERK) cascade, which also plays a critical role in T cell development and activation (Cantrell, 1996; Crompton et al., 1996). This is demonstrated by the observation that introduction of a gene coding for activated Ras drives the transition from DN to DP cells in RAG-2-deficient mice (Swat et al., 1996) and that antigen-unresponsive anergic T cells have a block in the Ras/MAPK/ERK pathway and fail to produce IL-2 (Fields et al., 1996). Thus, SLP-76 may function as an adaptor molecule linking the TCR, through Lck and ZAP-70, to the activation of the JNK and Ras pathways.

In an effort to understand the role of SLP-76 in T

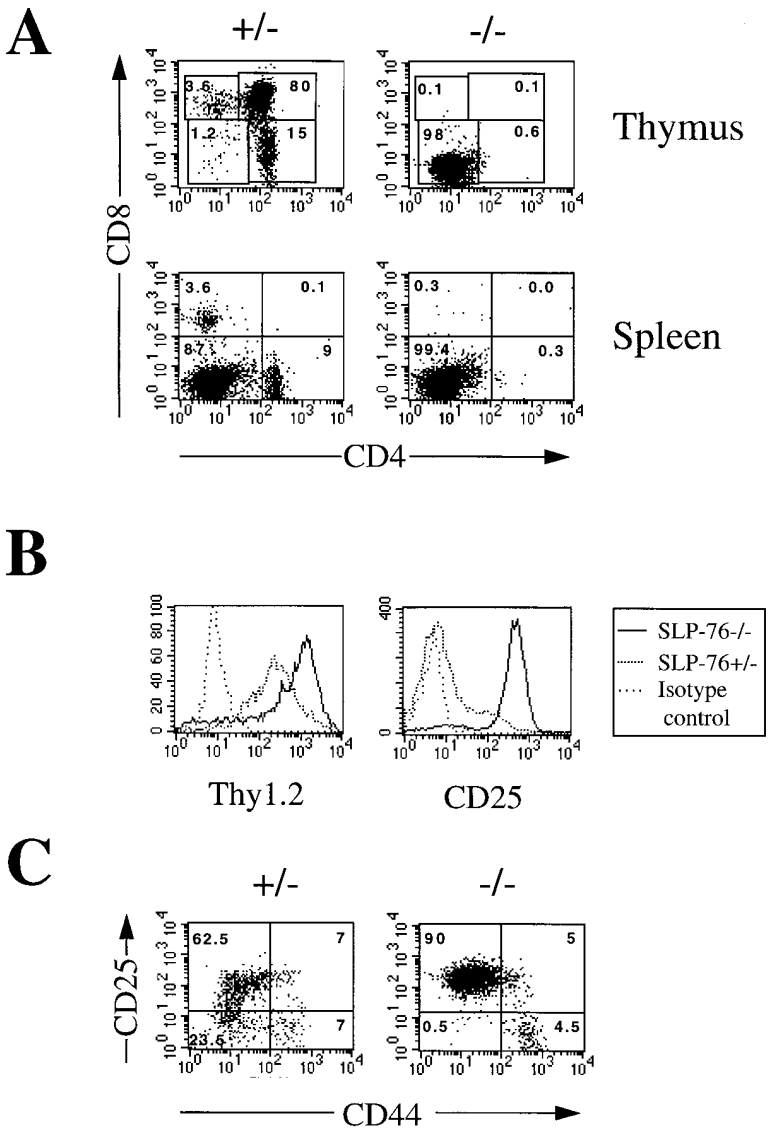

Figure 2. Flow Cytometry Analysis of Thymus and Spleen Cells from 3- to 4-Week-Old *SLP-76*$^{-/-}$ and *SLP-76*$^{+/-}$ Littermates

(A) Surface expression of CD4 (anti-CD4-FITC) versus CD8 (anti-CD8-PE) on thymocytes and on spleen cells.

(B) Surface expression of Thy-1 (anti-Thy.1.2-FITC) and CD25 (anti-CD25-PE) on thymocytes from *SLP-76*$^{-/-}$ and *SLP-76*$^{+/-}$ littermates.

(C) Surface expression of CD44 and CD25 on DN thymocytes from *SLP-76*$^{-/-}$ and *SLP-76*$^{+/-}$ littermates. Cells were triple stained with anti-CD44-FITC, anti-CD25-PE, and a cocktail of biotin-conjugated mAbs to CD3, CD4, CD8, B220, Mac1, and Gr-1, followed by avidin-CY-Chrome. Analysis was performed on gated CY-Chrome negative cells. The percentage of cells found in each quadrant is indicated.

In all FACS analyses (Figures 2–7), the results from *SLP-76*$^{+/-}$ and WT mice were similar. Therefore, only data on *SLP-76*$^{+/-}$ mice are shown.

cell development and function, we have generated mice homozygous for targeted disruption of the *SLP-76* gene. SLP-76-deficient mice had a profound block in thymic development with complete absence of DP cells. In contrast to RAG- and TCRβ-deficient mice, the block in the transition from DN to DP thymocytes in SLP-76-deficient mice could not be overcome by in vivo treatment with anti-CD3ε MAb. These results indicate that SLP-76 collects all pre-TCR signals that drive the development and expansion of DP thymocytes.

Results

Generation of SLP-76-Deficient Mice

The exon in the *SLP-76* gene that contains the translation initiation codon was replaced by a neomycin resistance gene in embryonic stem (ES) cells (Figure 1A). Three such targeted clones (Figure 1B) were used to establish mouse strains carrying the mutation. Heterozygous mice (*SLP-76*$^{+/-}$) were intercrossed to generate homozygous mutants (*SLP-76*$^{-/-}$) (Figure 1C). Disruption of the *SLP-76* gene was confirmed by demonstrating that thymocytes from *SLP-76* deficient mice, in contrast to CD25^{+} thymocytes from *SLP-76*$^{+/-}$ littermates, have no detectable SLP-76 protein (Figure 1D). This suggests that the engineered deletion blocks SLP-76 expression and can therefore be considered a null mutation.

At birth, *SLP-76*$^{-/-}$ pups showed subcutaneous bleeding. A large number of these pups died shortly (within a week) after birth. Of 85 mice analyzed at 4 weeks of age, only 8 were *SLP-76*$^{-/-}$. *SLP-76*$^{-/-}$ mice that survived had swollen footpads, slightly reduced body weight (~10% less than *SLP-76*$^{+/-}$ and wild-type [WT] littermates), and were found on autopsy to have bleeding in the peritoneum. Analysis of blood from *SLP-76*$^{-/-}$ mice showed some decrease in platelet numbers (30%–40%), compared to *SLP-76*$^{+/-}$ and WT littermates, which is consistent with reported expression of SLP-76 in platelets (Robinson et al., 1996). Although SLP-76 is normally

expressed in other myeloid cells (Jackman et al., 1995), analysis of blood from *SLP-76*$^{-/-}$ mice revealed no overt defects in the number of monocytes and granulocytes, as well as in blood clotting and erythrocyte maturation (data not shown).

T Cell Development in SLP-76-Deficient Mice Is Arrested at the DN Stage

The size and cellularity of the thymus were normal in *SLP-76*$^{+/-}$ mice. The thymus of *SLP-76*$^{-/-}$ mice was dramatically reduced in size, resembling that of RAG-deficient mice, and its cellularity was severely reduced to ~1% of that of heterozygous littermates. There were $1.2 \pm 0.5 \times 10^6$ thymocytes (counted as described in Experimental Procedures) in the thymus of *SLP-76*$^{-/-}$ mice, compared to $156 \pm 43 \times 10^6$ thymocytes in *SLP-76*$^{+/-}$ littermates (n = 4 for each group).

SLP-76$^{-/-}$ mice had no detectable CD4$^+$CD8$^+$ DP, CD4$^+$8$^-$ SP, or CD4$^-$8$^+$ SP thymocytes by flow cytometry (Figure 2A, upper panel). B220$^-$Mac1$^-$GR$^-$CD3$^-$ CD4$^-$CD8$^-$ DN thymocytes were present in *SLP-76*$^{-/-}$ mice, although their absolute number was slightly decreased. The number of these cells was $0.7 \pm 0.3 \times 10^6$ cells in *SLP-76*$^{-/-}$ thymus versus $1.88 \pm 0.5 \times 10^6$ cells in *SLP-76*$^{+/-}$ thymus (n = 4). These findings suggest an early block in thymocyte maturation. Consistent with such a block, there were virtually no detectable CD4$^+$ or CD8$^+$ SP cells in spleens of *SLP-76*$^{-/-}$ mice (Figure 2A, lower panel).

Thymocytes from *SLP-76*$^{-/-}$ mice expressed high levels of CD25 and Thy-1, characteristic of immature thymocytes, compared to thymocytes from *SLP-76*$^{+/-}$ littermates (Figure 2B). Analysis of the DN compartment for the expression of CD25 and CD44, which is used to define four developmental stages of thymocytes (CD25$^-$ CD44$^+$→CD25$^+$CD44$^+$→CD25$^+$CD44$^-$→CD25$^-$CD44$^-$) (Godfrey et al., 1993), showed a relative increase of the CD25$^+$CD44$^-$ population, whereas CD25$^-$CD44$^-$ cells, the most mature among DN T cell progenitors, were almost undetectable (Figure 2C). These results indicate that thymocyte development in *SLP-76*$^{-/-}$ mice does not proceed beyond the CD25$^+$44$^-$ stage.

TCRβ rearrangement normally occurs at the CD25$^+$ CD44$^-$ stage (Shortman and Wu, 1996). To evaluate the impact of SLP-76 deficiency on TCRβ rearrangements, we performed a DNA–PCR assay, using PCR primers for either Dβ1 or Vβ14 in combination with a Jβ primer 3′ of Jb1.2 (Gu et al., 1997). Figure 3 shows that both TCRβ D-J and V-D-J rearrangements were detectable in thymic DNA of *SLP-76*$^{-/-}$ mice. As expected, the TCRβ locus was not rearranged in control *RAG-2*$^{-/-}$ thymocytes. These findings indicate that SLP-76 is not essential for TCRβ rearrangement and suggest that *SLP-76*$^{-/-}$ mice may assemble a pre-TCR. Indeed, a small proportion (4%) of thymocytes from *SLP-76*$^{-/-}$ mice showed low expression of both CD3ε and TCRβ (Figure 4A). As expected, expression of TCRβ and CD3ε was undetectable on thymocytes from *RAG-2*$^{-/-}$ mice. Rearrangement of the TCRβ locus and surface expression of TCRβ and CD3ε in *SLP-76*$^{-/-}$ thymocytes argue that their developmental arrest at the DN stage was not due to failure to express a TCRβ/CD3ε–containing pre-TCR complex.

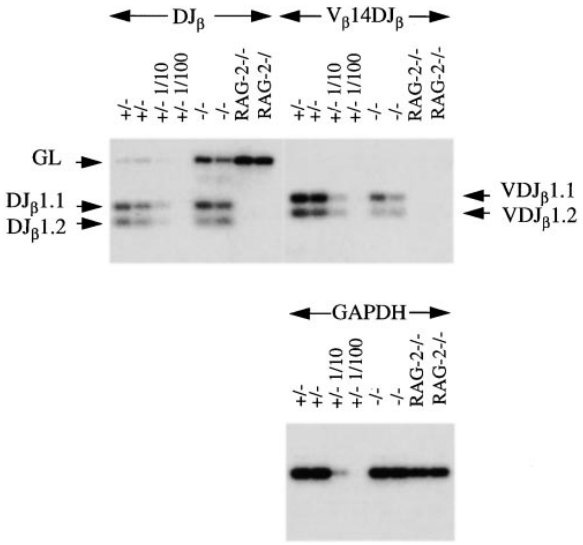

Figure 3. SLP-76-Deficient Thymocytes Rearrange TCRβ Genes

Detection of DJ and V(D)J rearrangement in TCRβ locus. Two hundred nanograms of *SLP-76*$^{+/-}$ (one sample diluted 1:10 and 1:100), 400 ng of *SLP-76*$^{-/-}$, and *RAG-2*$^{-/-}$ thymus DNA was assayed for TCRβ rearrangement. For DJ rearrangements, the PCR products that represent Dβ1-Jβ1.1 and Dβ1-Jβ1.2 rearrangements (350 and 230 bp, respectively) are evident in *SLP-76*$^{-/-}$ thymic DNA; only a germline band of 1.1 kb is detected in *RAG-2*$^{-/-}$ thymic DNA. For V(D)J rearrangement, the PCR products that represent Vβ14-Dβ1Jβ1.1 and Vβ14-Dβ1Jβ1.2 are detected in *SLP-76*$^{-/-}$ thymic DNA; no germline band is detected in *RAG-2*$^{-/-}$ thymic DNA because of the large distance that separates the primers in the unrearranged locus. Amplification of the murine glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) provides a control for amounts of loaded DNA.

The proportion of thymocytes that express γδTCR was relatively increased in *SLP-76*$^{-/-}$ mice compared to *SLP-76*$^{+/-}$ littermates: 3% versus 0.5% (Figure 4B; data not shown). However, the absolute number of γδTCR$^+$ thymocytes was considerably less in *SLP-76*$^{-/-}$ mice than in *SLP-76*$^{+/-}$ controls ($0.034 \pm 0.012 \times 10^6$ versus $0.52 \pm 0.11 \times 10^6$). These results suggest that development of γδ T cells is blocked in SLP-76-deficient mice. Consistent with such a block is the finding that CD3$^+$γδTCR$^+$ cells were undetectable in spleens of *SLP-76*$^{-/-}$ mice (Figure 4C).

Anti-CD3 MAb Fails to Drive the Transition from DN to DP Cells in SLP-76-Deficient Mice

Antibodies to CD3 induce DN thymocytes to differentiate into DP cells. This response does not require surface expression of TCRβ or pTα chains (Levelt et al., 1993; Shinkai and Alt, 1994) and is dependent on transduction of signals by Lck and Ras because introduction of transgenes that encode for constitutively active forms of Lck or Ras in *RAG-2*$^{-/-}$ and *pTα*$^{-/-}$ mice induces the transition from DN to DP cells (Swat et al., 1996; Fehling et al., 1997). We injected *SLP-76*$^{-/-}$ mice intraperitoneally with the anti-CD3ε mAb 2C11 and examined the expression of CD4 and CD8 on thymocytes 6 days following injection. As expected, anti-CD3ε induced differentiation and expansion of DP thymocytes when injected in *RAG-2*$^{-/-}$ controls (Figure 5A). The total number

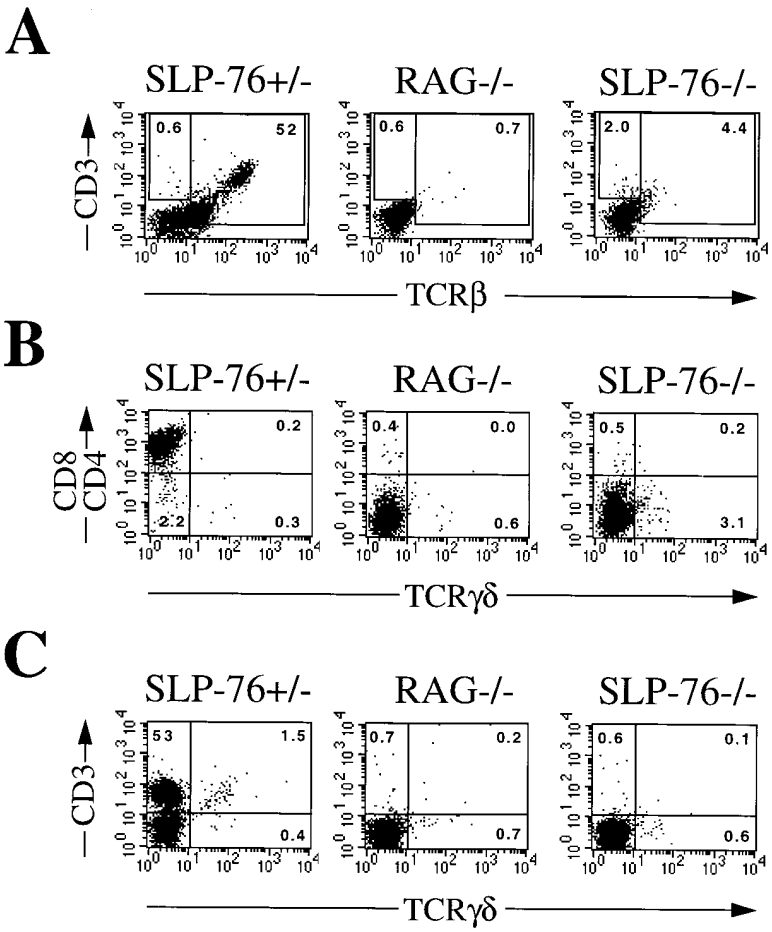

Figure 4. TCRβ and γδTCR Expression on Thymus and Spleen Cells from 3- to 4-Week-Old *SLP-76*$^{-/-}$ and *SLP-76*$^{+/-}$ Littermates and Age-Matched *RAG-2*$^{-/-}$ Controls

(A) Surface expression of CD3 (anti-CD3ε-PE) versus TCRβ (anti-TCRβ-FITC) and (B) γδTCR (anti-γδTCR-FITC) versus CD4 and CD8 (both biotinylated followed by streptavidin-PE) on thymocytes. (C) Surface expression of CD3 (anti-CD3ε-PE) versus γδTCR (anti-γδTCR-FITC) on spleen cells. The percentage of cells found in each quadrant is indicated.

of cells in the thymus of *RAG-2*$^{-/-}$ mice treated with anti-CD3 increased >10-fold ($0.72 \pm 0.31 \times 10^6$ cells in untreated mice, compared to 31.2×10^6 and 22.3×10^6 cells in two injected mice), and the percentage of DP cells exceeded 90%. In contrast, treatment of *SLP-76*$^{-/-}$ mice with anti-CD3ε mAb caused little if any increase in the number of thymocytes ($1.2 \pm 0.5 \times 10^6$ [n = 4] cells in untreated mice versus 2.7×10^6 and 2.53×10^6 cells in two treated mice) and induced few DP cells (<10%) (Figure 5A). Furthermore, anti-CD3 treatment induced the appearance of $CD44^-CD25^-$ cells in the DN population of *RAG-2*$^{-/-}$ thymocytes, whereas the response in *SLP-76*$^{-/-}$ mice was less pronounced. More than 30% of DN thymocytes became $CD44^-CD25^-$ in anti-CD3-treated *RAG-2*$^{-/-}$ mice versus ~10% in *SLP-76*$^{-/-}$ mice. These results suggest that SLP-76 plays a critical role in pre-TCR signaling that drives the development and expansion of DP thymocytes.

SLP-76$^{-/-}$ Mice Have Normal Numbers of Macrophages and NK Cells and Normal B Cell Development and Function

Expression of SLP-76 in NK cells has not been previously investigated. However, since NK cells have common precursors with thymocytes, it was important to determine whether the development of NK cells is impaired in *SLP-76*$^{-/-}$ mice. Figure 6A shows that *SLP-76*$^{-/-}$ mice have normal numbers of cells that express the NK marker DX5. Although human SLP-76 is expressed in monocytic cell line U-937 (Jackman et al., 1995), spleens from *SLP-76*$^{-/-}$ mice had normal numbers of $Mac1^+$ cells (Figure 6B). These results indicate that SLP-76 is not essential for the maturation of macrophage and NK cell lineages.

SLP-76$^{-/-}$ mice had normal cellularity and normal numbers of $B220^+$ (B) cells in the bone marrow and spleen (data not shown). Bone marrow cells from *SLP-76*$^{-/-}$ mice had a normal profile of staining for B220, IgM, and CD43 (Figure 7A), suggesting that B cell development is not affected. Spleen cells from *SLP-76*$^{-/-}$ mice had normal expression of B220, sIgM, and sIgD (Figure 7B). Furthermore, B cells from *SLP-76*$^{-/-}$ mice proliferated normally to lipopolysaccharide (LPS), anti-CD40 mAb, and anti-IgM, with or without IL-4 (data not shown). These results suggest that SLP-76 is not important for B cell development and proliferation in response to ligation of the B cell receptor (BCR) and CD40. This is consistent with recent data that indicates that normal B cells do not express SLP-76 (Fu and Chan, 1997).

Discussion

The reason for the impaired viability and hemorrhaging of *SLP-76*$^{-/-}$ mice is not known. The gross anatomy of the organs in these mice is normal except for bleeding,

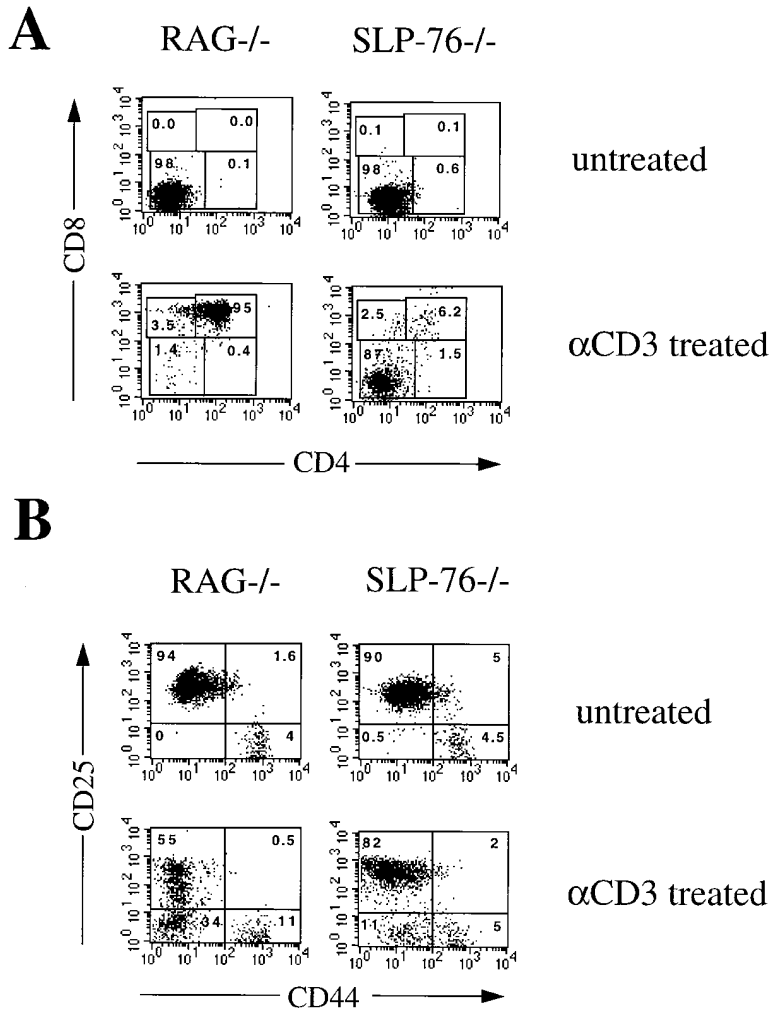

Figure 5. SLP-76-Deficient DN Thymocytes Do Not Progress to the DP Stage following In Vivo Treatment with anti-CD3 mAb

Flow cytometry analysis of thymocytes from *SLP-76*$^{-/-}$ and *RAG-2*$^{-/-}$ mice following anti-CD3 mAb treatment. Groups of two mice from *SLP-76*$^{-/-}$ and *RAG-2*$^{-/-}$ mice were injected intraperitoneally with 50 μg of anti-CD3ε mAb 2C11, and their thymocytes were analyzed 6 days later and compared to thymocytes from uninjected *SLP-76*$^{-/-}$ and *RAG-2*$^{-/-}$ mice (A) Surface expression of CD4 (anti-CD4-FITC) versus CD8 (anti-CD8-PE) on thymocytes from uninjected and injected mice. (B) Surface expression of CD25 (anti-CD25 -PE) and CD44 (anti-CD44-FITC) on DN thymocytes from uninjected and injected mice. The percentage of cells found in each quadrant is indicated.

suggesting that bleeding in vital organs may be responsible for the early death of *SLP-76*$^{-/-}$ mice. Whether the hemorrhaging in *SLP-76*$^{-/-}$ pups results from platelet dysfunction or has another cause is unknown. *Syk*$^{-/-}$ mice (Cheng et al., 1995; Turner et al., 1995), but not *ZAP-70*$^{-/-}$ mice (Negishi et al., 1995), have impaired viability and hemorrhaging. This suggests that a pathway involving Syk and SLP-76 is important in preventing hemorrhaging and for normal survival.

Thymocytes from mice that lack SLP-76 had a complete block in the transition from the DN to the DP stage. More specifically, thymocyte development was arrested at the CD25^{+}CD44^{-} DN stage at which TCRβ rearrangement occurs, the pre-TCR is assembled, and signals delivered through the pre-TCR induce the differentiation and expansion of DP cells. The block in thymocyte development in *SLP-76*$^{-/-}$ mice was not due to lack of expression of the pre-TCR, as TCRβ rearrangement proceeded in these mice and their thymocytes expressed both the TCRβ and CD3ε components of the pre-TCR. This strongly suggests that SLP-76 is critical for pre-TCR signaling that drives the progression of thymocytes from the DN to the DP stage.

The block in differentiation of DP thymocytes in *SLP-76*$^{-/-}$ mice is complete and matches in severity that observed in RAG-1/2-or CD3ε-deficient mice, which fail to express a pre-TCR. In contrast, mice deficient in TCRβ (Mombaerts et al., 1992a), pTα (Fehling et al., 1995), Lck (Molina et al., 1992), and ZAP-70 (Negishi et al., 1995) have variable but incomplete blocks in the differentiation of DP thymocytes. The incomplete block in development of DP thymocytes in Lck-deficient mice is likely due to the fact that Lck function is partly redundant with that of other PTKs, particularly Fyn. This was first suggested by the observation that introduction of a transgene encoding for an inactive Lck mutant into WT mice caused a more complete block in the development of DP thymocytes than targeted disruption of *lck*, possibly because it competed with other PTKs for substrate binding (Levin et al., 1993). Fyn deficiency has no appreciable effect on the development of DP cells, although it severely impairs the development of SP cells (Appleby et al., 1992; Stein et al., 1992). However, mice doubly deficient in Lck and Fyn have complete arrest in the development of DP cells, similar to the one we observe in SLP-76-deficient mice (Groves et al., 1996; van Oers et al., 1996).

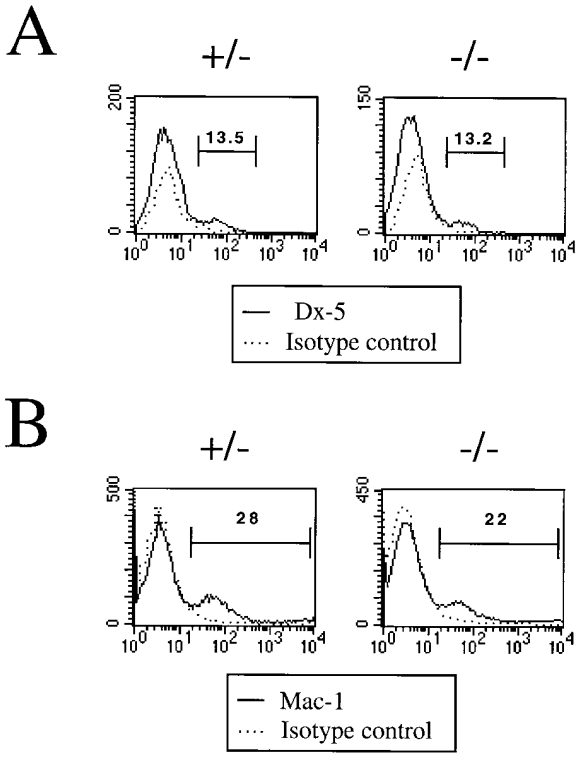

Figure 6. Flow Cytometry Analysis of Monocytes and NK in Spleen Cells of *SLP-76*$^{-/-}$ and *SLP-76*$^{+/-}$ Littermates

(A) Surface expression of the NK marker DX5 using anti-DX5-FITC. (B) Surface expression of Mac1 using biotinylated anti-Mac1 followed by streptavidin-CY.

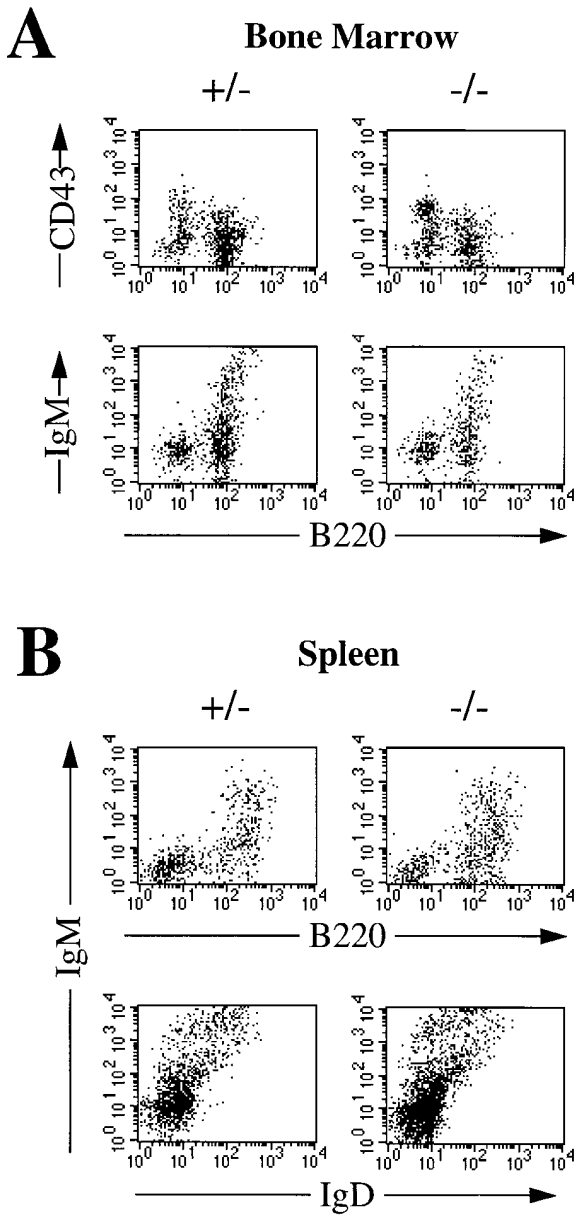

Figure 7. Flow Cytometry Analysis of B cells in Bone Marrow and Spleen Cells of *SLP-76*$^{-/-}$ and *SLP-76*$^{+/-}$ Littermates

(A) Expression of B220 (anti-B220-FITC) versus CD43 (anti-CD43-PE) and IgM (anti-IgM-bio-CYC) on bone marrow cells. (B) Expression of B220 (anti-B220-FITC) versus IgM (anti-IgM-bio-CYC), and IgM versus IgD (anti-IgD-FITC) on spleen cells.

Moreover, introduction of a gain-of-function *fyn* transgene in Lck-deficient mice completely restores the production of DP thymocytes (Groves et al., 1996). Thus, Lck and Fyn have overlapping functions in signaling from the pre-TCR.

Deficiency of ZAP-70 does not affect the development of DP thymocytes, although it impairs the development of SP cells in mice (Negishi et al., 1995) and of CD8$^+$ SP cells in humans (Arpaia et al., 1994). Deficiency of the ZAP-70-related kinase Syk has no effect on the development of TCR$\alpha\beta$ cells (Cheng et al., 1995; Turner et al., 1995). However, mice deficient both in ZAP-70 and Syk are arrested in their development at the DN stage (Cheng et al., 1997). Thus, ZAP-70 and Syk tyrosine kinases, like Lck and Fyn, have crucial but overlapping functions in signaling from the pre-TCR and in the maturation of DN thymocytes into DP cells. SLP-76 is a substrate for both ZAP-70 and Syk kinases. A likely explanation for the complete block in the transition from DN to DP cells in SLP-76-deficient mice is that all pre-TCR signals that drive the maturation of DN thymocytes into DP thymocytes converge on SLP-76.

Although it has not been previously investigated whether $\gamma\delta$ T cells express SLP-76, deficiency in this adaptor protein resulted in severe reduction of the number of $\gamma\delta$TCR$^+$ thymocytes and in the absence of $\gamma\delta$ T cells in the spleen. This suggests that signals regulating expansion and/or migration of these cells are transduced through SLP-76.

A most important finding is the inability of anti-CD3ϵ treatment to overcome the thymocyte developmental block in SLP-76-deficient mice. This observation supports the critical and indispensable role of SLP-76 in pre-TCR signaling. Anti-CD3ϵ causes efficient maturation and expansion of DP cells in RAG- and TCRβ-deficient mice, with >10-fold increase in cell number with >90% of thymocytes becoming DP cells. In contrast, in

SLP-76-deficient mice treated with anti-CD3ε, the number of thymocytes remained practically unchanged, and less than 10% of the thymocytes became DP cells. The resistance of the block in the transition from DN to DP cells in SLP-76-deficient mice is more severe than the one in Lck-deficient mice. In Lck-deficient mice, treatment with anti-CD3ε causes an ~5-fold increase in cell number, with ~60% of thymocytes becoming DP cells (Levelt et al., 1995). It is unlikely that the failure of anti-CD3 to cause maturation of DP cells in SLP-76-deficient mice was due to induction of apoptosis, because anti-CD3ε-mediated apoptosis is thought to depend on DP and SP thymocytes, which were completely lacking in SLP-76-deficient mice.

TCRβ allelic exclusion is dependent on signaling via the CD3 subunit of the pre-TCR (Ardouin et al., 1998). However, allelic exclusion of the TCRβ locus is not completely compromised in Lck-deficient mice (Wallace et al., 1995), suggesting that pre-TCR signals other than those initiated by Lck are critical. Given the severity of the thymic maturation block in SLP-76-deficient mice, it would be of interest to investigate TCRβ allelic exclusion in these mice by examining the effect of introduction of a TCRβ transgene on the rearrangement of the endogenous TCRβ loci and under more physiological conditions by single-cell PCR (Aifantis et al., 1997).

Since SLP-76 appears to stand at the crossroads of signaling pathways that originate from the pre-TCR, it will be important to define the role of signaling molecules that lie downstream from SLP-76 in the transition from DN to DP thymocytes. By virtue of its interaction with Vav and Grb2, SLP-76 may function as a scaffold, linking the pre-TCR to the activation of the JNK and Ras pathways. Since DP thymocytes develop in *Vav*$^{-/-}$ mice (Tarakhovsky et al., 1995; Zhang et al., 1995; Turner et al., 1997), pathways other than those that involve Vav are important for the transmission of signals downstream of SLP-76. All three domains of SLP-76 are required for optimal enhancement of IL-2 synthesis in mature T cells (Musci et al., 1997), suggesting that more than one signaling pathway is involved in SLP-76 induction of IL-2 gene transcription. The same could be true for pre-TCR-mediated induction of DP thymocytes. Introduction of transgenes encoding individual SLP-76 domains in SLP-76 mice will help determine the role of these domains and their associated molecules in SLP-76-mediated transduction of signals from the pre-TCR.

Although human SLP-76 is expressed in the monocytic cell line U-937 (Jackman et al., 1995), Mac-1$^+$ cells were present in normal numbers in spleens of SLP-76-deficient mice, suggesting that SLP-76 does not play a role in monocyte development. This is not surprising, because signaling via FcγR is not essential for monocyte development, as illustrated by normal numbers of Mac-1$^+$ in FcγRI-deficient mice (Takai et al., 1994). The effect of SLP-76 deficiency on the function of monocytes remains to be examined.

B cell development and proliferation in response to mitogenic stimuli that included LPS and ligation of the BCR and CD40 were not significantly affected in *SLP-76*$^{-/-}$ mice. Although SLP-76 has been reported to be expressed in B cell lines, little or no detectable levels are found in normal B cells. Given the strong parallels between the structure and function of the TCR and BCR, one would expect B cells to express an adaptor molecule analogous to SLP-76. Recent data indicate that B cells express SLP-76-related molecules that undergo intense tyrosine phosphorylation following ligation of the BCR and that associate with Grb2 (Fu and Chan, 1997).

Although the adaptor molecule SLP-76 is expressed in T lymphocytes and myeloid cells, its disruption selectively and severely impairs T cell development. This is probably because thymocyte maturation requires signaling via the pre-TCR that is absolutely dependent on the adaptor SLP-76 to relay signals from PTKs activated by the pre-TCR to downstream signaling cascades. Examination of the function of neutrophils, monocytes, and platelets in SLP-76-deficient mice and of T cell function in mice with conditional disruption of the *SLP-76* gene will establish whether SLP-76 functions differently in mature T and myeloid cells.

Experimental Procedures

Generation of SLP-76-Deficient Mice

The cDNA encoding murine SLP-76 was amplified by PCR from murine spleen cDNA using the 5′ primer ATGGCCTTGAAGAATGTC CCGTTTC and the 3′ primer CTACAGACAGCCTGCAGCGTG. The primers were designed based on the published murine *SLP-76* cDNA sequence (Jackman et al., 1995). DNA encoding the murine *SLP-76* gene was isolated from a Lambda FIXII library made from the 129Sv mouse strain (Stratagene, La Jolla, CA), using mouse *SLP-76* cDNA as a probe. DNA from isolated phages was purified and subjected to high-resolution restriction mapping by partial digestion and Southern blotting. The targeting construct was assembled in the pPNT targeting vector (Tybulewicz et al., 1991), using a 6.0 kb KpnI/KpnI fragment and a 3.0 kb SalI/EcoRI fragment, where the SalI site is from the multiple cloning site of FIXII. The targeting vector contains the neomycin resistance gene (*neo*), in the opposite direction to the *SLP-76* gene, for positive selection of the transfected ES cells, and a copy of the thymidine–kinase gene (*tk*) for the negative selection of spurious transformants that contain randomly integrated constructs. The construct (20 μg) was linearized by digestion at the unique NotI site in pPNT and used to transfect by electroporation 2×10^7 ES cells (J1) obtained from Dr. R. Jaenisch (MIT, Cambridge, MA). Transfected ES cells were selected in medium containing 0.4 mg/ml of G418 and 10 μg/ml of gancyclovir. Of 146 clones analyzed, 6 were identified to contain both a normal and a disrupted allele and no random integration of the *neo* gene. Five targeted ES clones were injected into 3.5-day-old C57BL/6 blastocysts, which were then transferred into Swiss foster mothers. Chimeric males were crossed with C57BL/6 females. Tail DNA of agouti offspring was analyzed by Southern blotting. *SLP-76* heterozygous (+/−) mice from the F1 generation were used to obtain *SLP-76* homozygous (−/−) mice by brother–sister mating. F2 offspring from these crosses were genotyped by Southern blotting to identify homozygotes.

Western Blot Analysis of SLP-76

Lysates from 1×10^6 thymocytes from *SLP-76*$^{-/-}$ mice and from 1×10^6 CD25$^+$ thymocytes from *SLP-76*$^{+/-}$ littermates were separated on a 4%–15% gradient gel by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and developed using affinity-purified goat polyclonal antibody against a peptide corresponding to amino acids 512–531, identical in human and mouse (Santa Cruz Biotechnology), followed by horseradish peroxidase–conjugated mouse anti-goat/sheep IgG (Sigma) and the Super Signal Ultra (Pierce) enhanced chemiluminescence system, according to manufacturer's instructions. CD25$^+$ thymocytes were isolated from single-cell suspension using anti-CD25 mAbs 7D4 (PharMingen) on

magnetic beads BioMag conjugated with goat anti-rat IgG (PerSeptive Diagnostics) and were found to be >90% CD25$^+$ by flow cytometry.

Antibodies and Flow Cytometry Analysis

Streptavidin-CY-Chrome, streptavidin-PE, and mAbs to the following mouse antigens were purchased from PharMingen: CD3ϵ, 145-2C11; CD4, L3T4; CD8α, 53-6.7; CD25, 7D4; CD44, IM7; Thy1.2, 53-2.1; Mac1, M1/70; Gr-1, RB6-8C5; B220, RA3-6B2; CD43, S7; CD40, 3/23; IgM, II/41; IgD, 11-26c.2a; $\gamma\delta$TCR, GL3; and TCRβ, H57-597. Anti-CD25 mAbs (clone 3C7) conjugated with phycoerythrin were purchased from Sigma. Single-cell suspensions from thymus and spleen were prepared by compression of lymphoid organs between ground glass slides, followed by centrifugation on density gradient of Lympholyte-M (Cedarlane). Total cell numbers were determined by counting cells using hemacytometer following centrifugation. Cells were stained and analyzed on a FACSCalibur flow cytometer (Becton Dickinson) as previously described (Hollander et al., 1996). Data on 5–20 $\times$ 10^5 viable nonerythroid cells (as determined by forward versus side scatter) were collected for each sample. FACS analysis was performed on cells from groups of at least three mice aged 2–4 weeks. The absolute cell numbers were calculated by multiplying the absolute frequency of each subset by the total cell count as determined above.

PCR Analysis of V(D)J Rearrangement

The PCR primers and conditions for detecting Dβ1-Jβ1.1 and Dβ1-Jβ.2 TCR and for amplifying GAPDH were as described previously (Gu et al., 1997). Two hundred nanograms of *SLP-76*$^{+/-}$ (one sample diluted 1:10 and 1:100), 400 ng of *SLP-76*$^{-/-}$, and *RAG-2*$^{-/-}$ thymus DNA was assayed for TCRβ rearrangement. For detection of Vβ14-Dβ1Jβ1.1 and Vβ14-Dβ1Jβ1.2 rearrangements, a Vβ14 5′ oligonucleotide (5′-ACCCTCCAGCAACTCTTC-3′) and the 3′ Jβ1.2 oligonucleotide described in Gu et al. (1997) were used under the following PCR conditions: preheat 3 min at 94°C, then 94°C, 1.5 min, 60°C, 2.5 min, 72°C, 1 min, for 35 cycles with 10 min polishing step. Amplified products were separated on a 1.1% Tris-buffered agarose gel, transferred onto a Zeta-probe membrane (Bio-Rad Laboratories), and hybridized with the Jβ2 probe 5′-AAAGCCTGGTCCCTGAGCCGA-3′ for Dβ-Jβ and Vβ-DβJβ rearrangements or a rat GAPDH cDNA probe for the DNA control sample (Gu et al., 1997).

Acknowledgments

This work was supported by NIH grant AI-35714; by grants from Baxter Healthcare, Alpha Therapeutics, and Olsten Corporations to R. S. G.; and a grant from the Howard Hughes Medical Institutes (F. W. A.).

Received April 10, 1998; revised June 9, 1998.

References

Aifantis, I., Buer, J., von Boehmer, H., and Azogui, O. (1997). Essential role of the pre-T cell receptor in allelic exclusion of the T cell receptor beta locus. Immunity *7*, 601–607.

Appleby, M.W., Gross, J.A., Cooke, M.P., Levin, S.D., Qian, X., and Perlmutter, R.M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59*fyn*. Cell *70*, 751–763.

Ardouin, L., Ismaili, J., Malissen, B., and Malissen, M. (1998). The CD3-gammadeltaepsilon and CD3-zeta/eta modules are each essential for allelic exclusion at the T cell receptor beta locus but are both dispensable for the initiation of V to (D)J recombination at the T cell receptor-beta, -gamma, and -delta loci. J. Exp. Med. *187*, 105–116.

Arpaia, E., Shahar, M., Dadi, H., Cohen, A., and Roifman, C.M. (1994). Defective T cell receptor signaling and CD8$^+$ thymic selection in humans lacking Zap-70 kinase. Cell *76*, 947–958.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Annu. Rev. Immunol. *14*, 259–274.

Cheng, A.M., Rowley, B., Pao, W., Hayday, A., Bolen, J.B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. Nature *378*, 303–306.

Cheng, A.M., Negishi, I., Anderson, S., Chan, A., Bolen, J., Loh, D., and Pawson, T. (1997). The Syk and ZAP-70 SH2-containing tyrosine kinases are implicated in pre-T cell receptor signaling. Proc. Natl. Acad. Sci. USA *94*, 9797–9801.

Crespo, P., Schuebel, K.E., Ostrom, A.A., Gutkind, J.S., and Bustelo, X.R. (1997). Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature *385*, 169–172.

Crompton, T., Gilmour, K.C., and Owen, M.J. (1996). The MAP kinase pathway controls differentiation from double-negative to double-positive thymocyte. Cell *86*, 243–251.

da Silva, A.J., Li, Z., de Vera, C., Canto, E., Findell, P., and Rudd, C.E. (1997a). Cloning of a novel T-cell protein FYB that binds FYN and SH2-domain-containing leukocyte protein 76 and modulates interleukin 2 production. Proc. Natl. Acad. Sci. USA *94*, 7493–7498.

da Silva, A.J., Raab, M., Li, Z., and Rudd, C.E. (1997b). TcR zeta/CD3 signal transduction in T-cells: downstream signaling via ZAP-70, SLP-76 and FYB. Biochem. Soc. Trans. *25*, 361–366.

Fehling, H.J., Krotkova, A., Saint-Ruf, C., and von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor α gene in development of $\alpha\beta$ but not $\gamma\delta$ T cells. Nature *375*, 795–798.

Fehling, H.J., Iritani, B.M., Krotkova, A., Forbush, K.A., Laplace, K., Perlmutter, R., and von Boehmer, H. (1997). Restoration of thymopoiesis in pTα−/− mice by anti-CD3ϵ antibody treatment or with transgenes encoding activated LCK or tailless pTα. Immunity *6*, 703–714.

Fields, P.E., Gajewsky, T.F., and Fitch, F.W. (1996). Blocked Ras activation in anergic CD4$^+$ T cells. Science *50*, 1276–1278.

Fu, C., and Chan, A.C. (1997). Identification of two tyrosine phosphoproteins, pp70 and pp68, which interact with phospholipase C-γ, Grb2, and Vav after B cell antigen receptor activation. J. Biol. Chem. *272*, 27362–27368.

Godfrey, D.I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3$^-$CD4$^-$CD8$^-$ triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J. Immunol. *150*, 4244–4253.

Groves, T., Smiley, P., Cooke, M.P., Forbush, K., Perlmutter, R.M., and Guidos, C. (1996). Fyn can partially substitute for Lck in T lymphocyte development. Immunity *5*, 417–428.

Gu, Y., Seidl, K.J., Rathbun, G.A., Zhu, C., Manis, J.P., van der Stoep, N., Davidson, L., Cheng, H.L., Sekiguchi, J.M., Frank, K., et al. (1997). Growth retardation and leaky SCID phenotype of Ku70-deficient mice. Immunity *7*, 653–665.

Hollander, G., Castigli, E., Kulback, R., Su, M., Burakoff, S., Gutierrez-Ramos, J.-C., and Geha, R. (1996). Induction of alloantigen-specific tolerance by B cells from CD40 deficient mice. Proc. Natl. Acad. Sci. USA *93*, 4994–4998.

Jackman, J.K., Motto, D.G., Sun, Q., Tanemoto, M., Turck, C.W., Peltz, G.A., Koretzky, G.A., and Findell, P.R. (1995). Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. J. Biol. Chem. *270*, 7029–7032.

Koretzky, G. (1997). The role of Grb2-associated proteins in T-cell activation. Immunol. Today *18*, 401–406.

Levelt, C.N., Mombaerts, P., Iglesias, A., Tonegawa, S., and Eichmann, K. (1993). Restoration of early thymocyte differentiation in T-cell receptor β-chain-deficient mutant mice by transmembrane signaling through CD3e. Proc. Natl. Acad. Sci. USA *90*, 11401–11406.

Levelt, C.N., Mombaerts, P., Wang, B., Kohler, H., Tonegawa, S., Eichmann, K., and Terhorst, C. (1995). Regulation of thymocyte development through CD3: functional dissociation between p56*lck* and CD3ζ in early thymic selection. Immunity *3*, 215–222.

Levin, S.D., Anderson, S.J., Forbush, K.A., and Perlmutter, R.M. (1993). A dominant-negative transgene defines a role for p56^{lck} in thymopoiesis. EMBO J. *12*, 1671–1680.

Malissen, M., Gillet, A., Rocha, B., Trucy, J., Vivier, E., Boyer, C., Kontgen, F., Brun, N., Mazza, G., Spanopoulou, E., et al. (1993). T cell development in mice lacking the CD3-zeta/eta gene. EMBO J. *12*, 4347–4355.

Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E., and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3- epsilon gene. EMBO J. *14*, 4641–4653.

Molina, T.J., Kishihara, K., Siderovski, D.P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.-U., Veilette, A., et al. (1992). Profound block in thymocyte development in mice lacking p56^{lck}. Nature *357*, 161–164.

Mombaerts, P., Clarke, A.R., Rudnicki, M.A., Iacomini, J., Itohara, S., Lafaille, J.J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M.L., et al. (1992a). Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. Nature *360*, 225–231.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992b). RAG-1 deficient mice have no mature B and T lymphocytes. Cell *68*, 869–877.

Mombaerts, P., Anderson, S.J., Perlmutter, R.M., Mak, T.W., and Tonegawa, S. (1994). An activated form of Ick transgene promotes thymic development in RAG-1 mutant mice. Immunity *1*, 261–268.

Motto, D.G., Ross, S.E., Wu, J., Hendricks-Taylor, L.R., and Koretzky, G.A. (1996). Implication of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production. J. Exp. Med. *183*, 1937–1943.

Musci, M.A., Motto, D.G., Ross, S.E., Fang, N., and Koretzky, G.A. (1997). Three domains of SLP-76 are required for its optimal function in a T cell line. J. Immunol. *159*, 1639–1647.

Negishi, I., Motoyama, N., Nakayama, K.-I., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A.C., and Loh, D.Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature *376*, 435–438.

Raab, M., da Silva, A.J., Findell, P.R., and Rudd, C.E. (1997). Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCR zeta/CD3 induction of interleukin-2. Immunity *6*, 155–164.

Robinson, A., Gibbins, J., Rodriguez-Linares, B., Finan, P.M., Wilson, L., Kellie, S., Findell, P., and Watson, S.P. (1996). Characterization of Grb2-binding proteins in human platelets activated by Fc gamma RIIA cross-linking. Blood *88*, 522–530.

Shinkai, Y., and Alt, F. (1994). CD3ϵ-mediated signals rescue the development of CD4$^+$CD8$^+$ thymocytes in RAG-2$^{-/-}$ mice in the absence of TCRb chain expression. Int. Immunol. *6*, 995–1001.

Shinkai, Y., Koyasu, S., Nakayama, K., Murphy, K.M., Loh, D.Y., Reinherz, E.L., and Alt, F.W. (1992a). Restoration of T cell development in RAG2-deficient mice by functional TCR transgenes. Science *259*, 822–825.

Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., et al. (1992b). RAG-2 deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855–867.

Shortman, K., and Wu, L. (1996). Early T lymphocyte progenitors. Annu. Rev. Immunol. *14*, 29–47.

Stein, P.L., Lee, H.-M., Rich, S., and Soriano, P. (1992). pp59*fyn* mutant mice display differential signaling in thymocytes and peripheral T cells. Cell *70*, 741–750.

Swat, W., Shinkai, Y., Cheng, H.-W., Davidson, L., and Alt, F.W. (1996). Activated Ras signals differentiation and expansion of CD4$^+$8$^+$ thymocytes. Proc. Natl. Acad. Sci. USA *93*, 4683–4687.

Takai, T., Li, M., Sylvestre, D., Clynes, R., and Ravetch, J.V. (1994). FcR γ chain deletion results in pleiotrophic effector cell defects. Cell *76*, 519–529.

Tarakhovsky, A., Turner, M., Schaal, S., Mee, P.J., Duddy, L.P., Rajewsky, K., and Tybulewicz, V.L.J. (1995). Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. Nature *374*, 467–470.

Tuosto, L., Michel, F., and Acuto, O. (1996). p95vav associates with tyrosine-phosphorylated SLP-76 in antigen-stimulated T cells. J. Exp. Med. *184*, 1161–1166.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L., and Tybulewicz, V.L.J. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature *378*, 298–302.

Turner, M., Mee, P.J., Walters, A.E., Quinn, M.E., Mellor, A.L., Zamoyska, R., and Tybulewicz, V.L.J. (1997). A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity *7*, 451–460.

Tybulewicz, V.L., Crawford, C.E., Jackson, P.K., Bronson, R.T., and Mulligan, R.C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the *c-abl* proto-oncogene. Cell *65*, 1153–1163.

van Oers, N.S.C., Lowin-Kropf, B., Finlay, D., Connolly, K., and Weiss, A. (1996). $\alpha\beta$ T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. Immunity *5*, 429–436.

von Boehmer, H., and Fehling, H.J. (1997). Structure and function of the pre-T cell receptor. Annu. Rev. Immunol. *15*, 433–452.

Wallace, V.A., Kawai, K., Levelt, C.N., Kishihara, K., Molina, T., Timms, E., Pircher, H., Penninger, J., Ohashi, P.S., Eichmann, K., et al. (1995). T lymphocyte development in p56lck deficient mice: allelic exclusion of the TcR beta locus is incomplete but thymocyte development is not restored by TcR beta or TcR alpha beta transgenes. Eur. J. Immunol. *25*, 1312–1318.

Wardenburg, J.B., Fu, C., Jackman, J.K., Flotow, H., Wilkinson, S.E., Williams, D.H., Johnson, R., Kong, G., Chan, A.C., and Findell, P.R. (1996). Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. J. Biol. Chem. *271*, 19641–19644.

Wu, J., Motto, D.G., Koretzky, G.A., and Weiss, A. (1996). Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity *4*, 593–602.

Zhang, R., Alt, F.W., Davidson, L., Orkin, S.H., and Swat, W. (1995). Defective signaling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. Nature *374*, 470–473.